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Research review paper

Ubiquitous Chromatin-opening Elements (UCOEs): Applications in biomanufacturing and gene therapy

Jonathan J. Neville[a], Joe Orlando[b], Kimberly Mann[b], Bethany McCloskey[a], Michael N. Antoniou[a,*]

[a] Gene Expression and Therapy Group, King's College London, Faculty of Life Sciences and Medicine, Department of Molecular Genetics, Guy's Hospital, London Bridge, London SE1 9RT, United Kingdom
[b] MilliporeSigma, Process Solutions, 80 Ashby Road, Bedford, MA 01730, USA

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ABSTRACT

Ubiquitous Chromatin-opening Elements (UCOEs) are defined by their ability to consistently confer stable, site of integration-independent transgene expression that is proportional to copy number, including from within regions of heterochromatin such as centromeres. UCOEs structurally consist of methylation-free CpG islands encompassing single or dual divergently-transcribed housekeeping gene promoters. Since their discovery in 1999, UCOEs and their sub-fragments have found applications in areas of biotechnology requiring stable, reproducible, and high levels of gene expression. This review recounts the discovery of UCOEs and examines their current and future applications in protein therapeutic biomanufacturing and gene therapy.

1. Introduction

The random integration of a transgene into a heterochromatic chromatin environment and the methylation of promoter DNA are major mechanisms that are antagonistic to gene expression, resulting in a variegated pattern of gene expression or silencing (Pikaart et al., 1998; Yang et al., 2010). Because stable and high level transgene expression are essential for the efficient and rapid production of clonal cell lines in biomanufacturing as well as for the lifelong expression of a transgene at a therapeutic level in gene therapy, there is a fundamental requirement for genetic regulatory elements, which can prevent gene silencing and maintain high levels of expression for long periods of time.

Genetic regulatory elements that confer a transcriptionally permissive state can be broadly dichotomised into those that actively function through dominant chromatin remodelling mechanisms and those that function as border or boundary elements to restrict the spread of heterochromatin marks into regions of euchromatin. The latter include insulators, scaffold/matrix attachment regions (S/MARs), and stabilising anti-repressor (STAR) elements, whilst the former comprise locus control regions (LCRs) and ubiquitous chromatin opening elements (UCOEs). LCRs and UCOEs are defined by their ability to consistently confer site of integration-independent stable transgene expression that is proportional to transgene copy number, even when integrated into heterochromatin (Antoniou et al., 2003; Li et al., 2002). LCRs are tissue-specific regulatory elements that consist of multiple subcomponents characterised by DNase I hypersensitivity and a high density of transcription factor binding sites (Kim and Dean, 2012; Li et al., 2002; Tam et al., 2006). In contrast, UCOEs function ubiquitously and neither consist of multiple DNase I hypersensitive sites that are characteristic of LCRs, nor are they required to flank a transgene at both 5′ and 3′ ends in order to exert their function as in the case of insulators and S/MARs (Antoniou et al., 2003; Williams et al., 2005). Thus, structurally and functionally UCOEs represent a distinct class of genetic regulatory element.

UCOEs have found widespread usage in protein therapeutic biomanufacturing applications as a means to manage costs and resources as well as to reliably expedite the generation of highly expressing recombinant cell clones. Similarly, UCOEs show great promise in the field of gene therapy by providing stable ubiquitous or tissue-specific expression in somatic tissues as well as in adult, embryonic, and induced pluripotent stem cells and their differentiated progeny. This review recounts the discovery of UCOEs, and discusses their application in biomanufacturing and gene therapy.

2. Discovery of ubiquitous chromatin-opening elements

The first genomic fragment found to possess a ubiquitous chromatin-opening function and protect against the epigenetic silencing of transgenes was derived from the TATA-binding protein (*TBP*) locus, a

* Corresponding author.
E-mail address: michael.antoniou@kcl.ac.uk (M.N. Antoniou).

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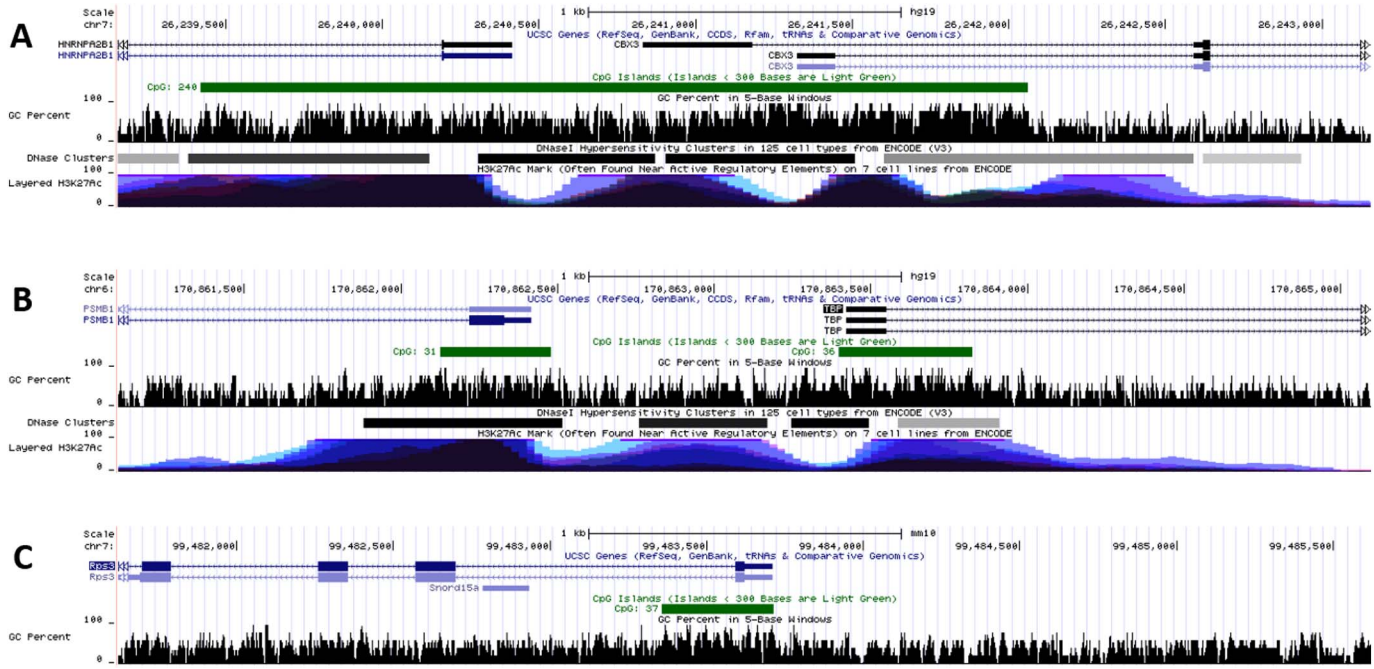

Fig. 1. The genomic organization of the prototypical ubiquitous chromatin opening elements (UCOEs). An overview of the (A) *HNRPA2B1-CBX3* A2UCOE, (B) *TBP-PSMB1* UCOE, and (C) *Rps3* UCOE regions, showing publicly available epigenomic data. CGI position (green bars with CpG count to their left), GC percentage (black columns each representing 5 bp; 0–100%), DNase I hypersensitivity sites (grey and black bars; the darkness of the bar reflects the assay signal intensity, with a darker bar indicating a stronger signal), and layered H3K27 acetylation marks from seven cell lines (each colour blue represents a cell line; density 0–100%). (A) The A2UCOE genomic region consisting of the closely-linked and divergently-transcribed first exon and promoter of *HNRPA2B1* and the alternate first exons and promoters of *CBX3*. (B) The *TBP-PSMB1* locus consisting of the closely-linked and divergently-transcribed first exons and promoters of *TBP* and *PSMB1*. (C) The murine *Rps3* locus. University of California, Santa Cruz (UCSC) genes track from the UCSC genome browser: http://genome.ucsc.edu/index.html. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

region encompassing the *TBP* and proteosomal subunit C5-encoding (*PSMB1*) housekeeping genes (Fig. 1A) (Harland et al., 2002). *TBP* and *PSMB1* are closely linked (within 1 kb) and divergently transcribed. Typical of housekeeping genes, *TBP* and *PSMB1* express ubiquitously and contain promoters encompassed by methylation-free CpG islands (CGI). Functional expression analysis of a 44 kb *TBP-PSMB1* fragment in stably transfected murine fibroblast L-cells after 60 days showed a level of *TBP* expression comparable to day zero, suggesting that this region protected the *TBP* promoter against silencing and enabled continuous and stable expression. In an effort to identify the location of regulatory elements of the *TBP-PSMB1* locus, DNase I hypersensitivity site mapping showed the presence of hypersensitive sites only at the *TBP* and *PSMB1* promoters (Harland et al., 2002). The authors concluded that the combination of stable *TBP* expression within a transgene context, together with DNase I hypersensitivity sites is structurally unlike that of previously identified LCRs (Li et al., 2002; Tam et al., 2006), indicating that a novel class of regulatory elements, which acts to negate epigenetic-mediated silencing and remodel chromatin structure, had been discovered.

Further functional expression analysis of the *TBP-PSMB1* locus was undertaken, together with a second region of similar genomic architecture, the *HNRPA2B1-CBX3* locus (Fig. 1B). The *HNRPA2B1-CBX3* locus consists of a region of closely-linked, divergently-transcribed housekeeping genes: heterologous nuclear ribonucleoprotein A2/B1 (*HNRPA2B1*) and heterochromatin protein 1Hs-γ (*CBX3*). A 2.6 kb methylation-free CGI (defined as such because it has a GC content of 61.5% and an observed-to-expected CpG of ratio of 0.97, meaning that this region is GC rich and contains a far greater density of CpG dinucleotides than would be expected by chance, overlies the first alternate exons of *CBX3* and the first exon of *HNRPA2B1* (Antoniou et al., 2003; Williams et al., 2005). These early functional studies showed that the *TBP-PSMB1* and *HNRPA2B1-CBX3* loci, including their dual divergently transcribed promoter regions, are able to dominantly open chromatin and confer stable gene expression as demonstrated by their ability to function even with transgene integration events into centromeric heterochromatin, with no observed position effect variegation (Antoniou et al., 2003), and thus represent the prototypical UCOEs.

The potential applications of UCOEs were first illustrated in 2005. Investigators linked fragments of the *HNRPA2B1-CBX3* methylation-free CGI and associated promoters (A2UCOE) to the immediate-early promoter-enhancer region of the human cytomegalovirus (*hCMV*). Expression from the *HNRPA2B1* and *CBX3* promoters is relatively low and the *hCMV* promoter region represents a non-selective, strong promoter-enhancer combination commonly used in mammalian cells to achieve high level expression of linked genes. It was observed that the linkage of various sized fragments (1.5 kb, 4 kb, and 8 kb) of the A2UCOE to the *hCMV* promoter resulted in a marked elevation of transgene expression and a resistance to silencing for up to 107–199 generations within stably transfected Chinese Hamster Ovary (CHO) K1 cells (Williams et al., 2005). This ability of the A2UCOE to confer a site of integration-independent, dominant chromatin-opening function and to protect linked transgenes from silencing showed great promise for biomanufacturing and gene therapy.

Structural similarities between the *TBP-PSMB1* UCOE and A2UCOE suggested potential functional mechanisms through common characteristics (Fig. 1). Both loci consisted of dual divergently-transcribed housekeeping genes with promoters encompassed by an extensive methylation-free CGI; it was therefore proposed that the chromatin remodelling capacity of UCOEs stemmed from the presence and combination of these features. Characterisation of the epigenetic signature of the native A2UCOE region in peripheral blood mononuclear cells revealed the coexistence of active histone H3 methylation and acetylation marks at the transcriptional start sites of *HNRPA2B1* and *CBX3*. Euchromatic histone H4 acetylation was also observed to be present throughout the A2UCOE region. However, overall histone modifications within the proximity of the A2UCOE were sparse due to nucleosome depletion (Lindahl Allen and Antoniou, 2007; Majocchi et al., 2014). Reduction of the levels of repressive histone marks

(H3K9me3 and H3K27me3), extensive areas of unmethylated DNA, and histone acetylation within the A2UCOE is thought to facilitate expression at the divergently-transcribed promoters and thus to initiate euchromatic remodelling (Majocchi et al., 2014). It was also proposed that a minimal functional region of the A2UCOE CGI was required for UCOE function (Lindahl Allen and Antoniou, 2007).

However, the discovery of the murine ribosomal protein S3 (*Rps3*) UCOE (Fig. 1C) cast doubt on the requirement for dual divergent transcription for UCOE function. In contrast to the *TBP-PSMB1* UCOE and the A2UCOE, the *Rps3* UCOE consists of a single housekeeping gene promoter element associated with a 358 bp CGI. A 3 kb *Rps3* element linked to the *hCMV* promoter produced high levels of enhanced green fluorescent protein (*EGFP*) expression at day 28, comparable to that of the *HNRPA2B1* promoter (Simpson et al., 2009). Thus the *Rps3* UCOE represents an effective UCOE containing a single housekeeping gene encompassed by a CGI.

3. Biomanufacturing applications of UCOEs

Modern biomedical research and many biotechnological techniques rely upon the efficient, cost-effective, robust production of large quantities of high quality protein in a short period of time. Tissue culture cell lines adapted to suspension growth in serum free media provide a scalable and easily purifiable source of therapeutic proteins. CHO cells are currently the most widely used mammalian host for recombinant protein production due to their well-established safety and simplicity of use (Jostock and Knopf, 2012; Wurm, 2004).

Benton and colleagues showed as early as 2002 that A2UCOE-regulated transgenes integrated into CHO sub-clones adapted to grow in suspension and survive in serum-free media (CHO-S), are able of rapidly producing large quantities of protein, with the production of up to 0.2 g/L of recombinant antibody (20 g produced in a 100 L bioreactor) obtained within five weeks of transfection (Benton et al., 2002). The presence of the A2UCOE also affords increased recombinant protein expression per vector copy number at reproducible levels, with small variation in expression within and between cell lines transfected with UCOE-containing vectors, compared to cells transfected with non-UCOE-containing vectors (Betts and Dickson, 2015).

Many biomanufacturing applications of UCOEs focus on the production of monoclonal antibodies (Betts et al., 2015; Betts and Dickson, 2015; Boscolo et al., 2012; Dharshanan et al., 2014; Hou et al., 2014). By comparing the efficiency of recombinant antibody production when using a 4 kb A2UCOE-based vector against a standard *hCMV* promoter-driven expression system in CHO-S cells, it was observed that the use of the A2UCOE-based vector system resulted in a higher overall level of expression of antibody relative to the standard *hCMV* promoter. Furthermore the A2UCOE-based vector showed a higher number of stably transfected clones, and outperformed constructs at medium-scale protein production levels where transgene expression was from the *hCMV* promoter alone, with antibody yields of 180–230 mg obtained per 1 L bioreactor flask (Boscolo et al., 2012). Similarly, upon comparison of an A2UCOE-based system against a commercially available immunoglobulin expression vector (pFUSE) for stability and expression levels of heavy and light chains of the humanised anti-C2 monoclonal antibody, the UCOE-based vector, upon transduction into preadapted serum-free CHO cells, produced a greater number of stable clones with recombinant antibody production levels of up to 50–110 mg/L (Dharshanan et al., 2014).

The period of time from initial transfection up to the identification and culture of a clonal cell population expressing a transgene at high levels is a rate-limiting step in recombinant antibody production. Hou and colleagues used the 3 kb Rps3 or the 8 kb A2UCOE linked to either a guinea pig *CMV* or *hCMV* promoter expressing IgG-1 and IgG-4 light and heavy monoclonal antibody chains to develop a rapid method for early-stage cell line development and clonal isolation in suspension-adapted CHO cells. The usage of UCOE-based vectors paired with robotic clone selection allowed the investigators to move from transfection to culture of high-expressing clones in only four weeks (Hou et al., 2014). Similarly, CHO cells stably transfected with UCOE-containing vectors expressing *EGFP* and erythropoietin (*EPO*) showed improved growth and survival, and retention of stable protein expression under selection with methotrexate, providing a means to identify and culture stably transfected cells (Betts et al., 2015; Betts and Dickson, 2015). The usage of UCOEs also resulted in fewer chromosomal rearrangements after selection with methotrexate, and greater homogeneity of chromosomal site integration over long-term culture compared to non-UCOE-containing vectors (Betts and Dickson, 2016).

In order to determine the most effective chromatin remodelling element for the production of monoclonal antibodies, Saunders and colleagues compared the function of a core 1.5 kb A2UCOE subfragment (Williams et al., 2005) to a series of border-type genetic regulatory elements, which function to block the spreading of repressive epigenetic marks: the MAR X_S29 S/MAR (Girod et al., 2007), the STAR40 element (Kwaks et al., 2003), and the chicken β-globin HS4 insulator (Barkess and West, 2012). These four chromatin remodelling elements were positioned at various locations relative to the heavy and light chain antibody transcription units driven by *hCMV* promoters within a single plasmid vector. Following the stable transfection and positive selection of CHO pools of cells, the A2UCOE-containing vector showed improved stability and greater than six-fold increase in antibody expression compared to the S/MAR, STAR, and insulator element containing vectors (Saunders et al., 2015).

Besides early successes in antibody production, UCOEs have shown efficacy in expressing other recombinant proteins. CHO cells transfected with a vector construct containing an 8 kb A2UCOE linked to a *hCMV*-EPO cassette were cultured at high cell density and resulted in the isolation of greater numbers of highly expressing clones, higher mean EPO production, and greater mRNA recovery per vector copy number compared to a non-UCOE containing vector (Betts and Dickson, 2015).

A comparison of the 3 kb *Rps3* UCOE and 1.5 kb A2UCOE core subfragment linked to seven different heterologous promoters expressing a large B-domain deleted factor VIII (BDD-FVIII) blood clotting factor gene, was undertaken in the baby hamster kidney (BHK) fibroblast cell line (Nair et al., 2011). Linked to all heterologous promoters tested, the A2UCOE showed greater levels of BDD-FVIII production and activity in adherent and serum-free suspension cultures. However, in CHO cells, the *Rps3* UCOE linked to *hCMV* was shown to outperform the A2UCOE, illustrating the importance of identifying the most effective UCOE-promoter combination for a given cell line (Simpson et al., 2009). A recent study systematically compared the effect of different factors on antibody production, including the use of UCOEs and promoters, in CHO-S cells (Rocha-Pizaña et al., 2017). The authors compared the use of double (*Rps3* and 1.5 kb A2UCOE) and single (*Rps3*) UCOE-containing vectors, and found that the double UCOE-based vector gave a higher level of antibody production. In addition, three different heterologous promoters linked to the *Rps3* UCOE were compared for their ability to drive expression of either the anti-TNFα or anti-CD20 + antibodies. The results varied, indicating that the most effective promoter is dependent on the protein being expressed. Furthermore, the order of heavy and light chain genes in the expression cassette was investigated and found to have no significant effect on antibody production (Rocha-Pizaña et al., 2017).

Experiments involving co-transfection of plasmid vectors harbouring separate light and heavy chain antibody cassettes under the control of the hCMV promoter with and without the A2UCOE, have also been reported (Nematpour et al., 2017). The results obtained indicate that including the A2UCOE as part of the heavy chain gene cassette is most crucial to obtaining increased and stable antibody output compared with constructs not containing the A2UCOE. (Nematpour et al., 2017).

UCOEs are available commercially for biomanufacturing and research applications in the form of ready-to-use plasmid vectors contain-

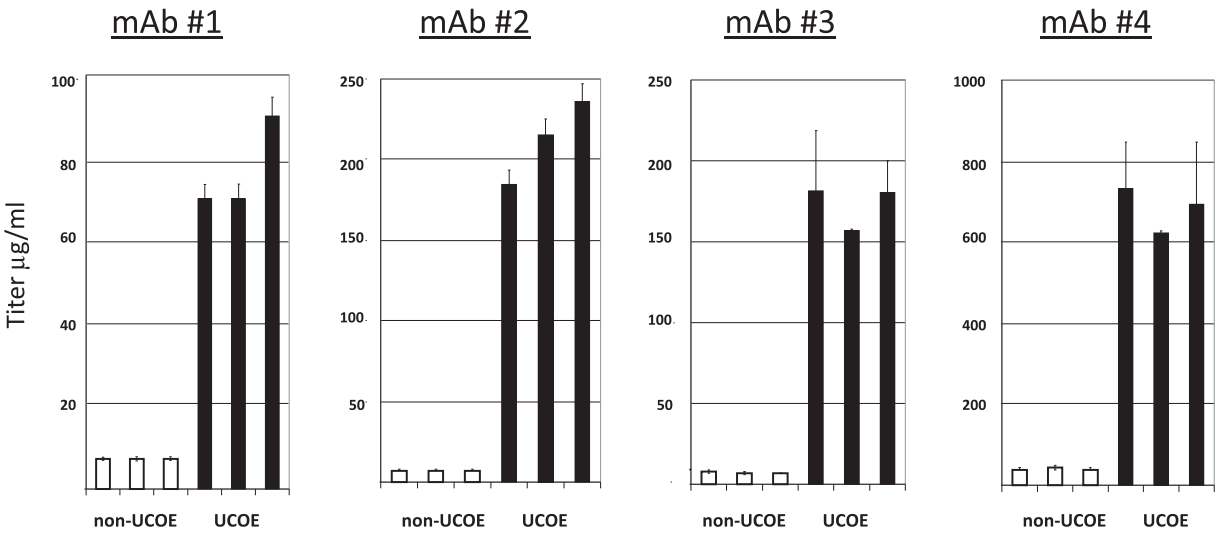

Fig. 2. UCOE enhances bulk titer monoclonal antibody expression. Four different monoclonal antibodies were cloned into MilliporeSigma commercial offering of single expression or dual plasmid expression vectors with and without the Rps3 UCOE. DNA was stably transfected into suspension-adapted CHO host cells by electroporation and cells selected for transgene integration by addition of puromycin and then subjected to antibody titer evaluation secreted into the batch culture medium. Titers were obtained using HPLC.

ing expression and selection elements and as DNA sequences that can be cloned into a vector of choice. An illustration of using the commercial UCOE vector offering is shown in Fig. 2. Here expression of four different monoclonal antibodies were compared in the presence and absence of the 3 kb Rps3 UCOE in different CHO cell-based hosts. For this purpose, triplicate flasks of cells were stably transfected, placed under selection in bulk and then analyzed in batch cultures. Although expression of the individual monoclonal antibodies differed across the panel, the inclusion of the Rps3 UCOE in the vector improved bulk stable pool expression by > 10-fold for each antibody (Fig. 2).

The inclusion of the Rps3 UCOE confers benefits in the cell line development process beyond increased monoclonal antibody titres (Fig. 2). Bulk Rps3-UCOE and non-UCOE vector-derived cell pools (Fig.2) were separated into single cell clones by limiting dilution and expanded in static cultures for two weeks. At that time, > 500 clones for UCOE and non-UCOE control cultures were evaluated for cell number and monoclonal antibody titer. Titer was divided by cell number to provide a relative expression level and the most productive 100 clones for each pool are shown in descending order (Fig. 3). Strikingly, the specific productivity of UCOE-derived clones dwarfs the collective productivity of the control clones. Furthermore, each of the 100 UCOE-derived clones has higher relative productivity than 98% of the control clones. The most productive 20 clones were selected for batch culture titer evaluation and the results of the top five UCOE-derived and control cultures are shown in the inset of Fig. 3. UCOE-derived clones produced as much as 0.7 g/L in the small scale batch evaluation whereas the control cultures produced < 0.1 g/L. Since the incorporation of the Rps3 UCOE aids in promoting stable transgene expression, fewer clones need to be screened to isolate high producers of the desired therapeutic protein. Moreover, the increased productivity of UCOE-derived clones alleviates the need to amplify the integrated transgenes as in DHFR CHO based systems (Gu et al., 1992; Kaufman and Sharp, 1982). Therefore, UCOE-based vectors provide benefits in the cell line development workflow including reduced resources required as well as potential time savings to isolate highly productive clones.

In summary, UCOEs confer reproducible and stable high levels of linked gene expression when stably transfected into serum-free, suspension-adapted, and adherent cell lines widely used in biomanufacturing applications. Furthermore, UCOEs have shown utility in the rapid

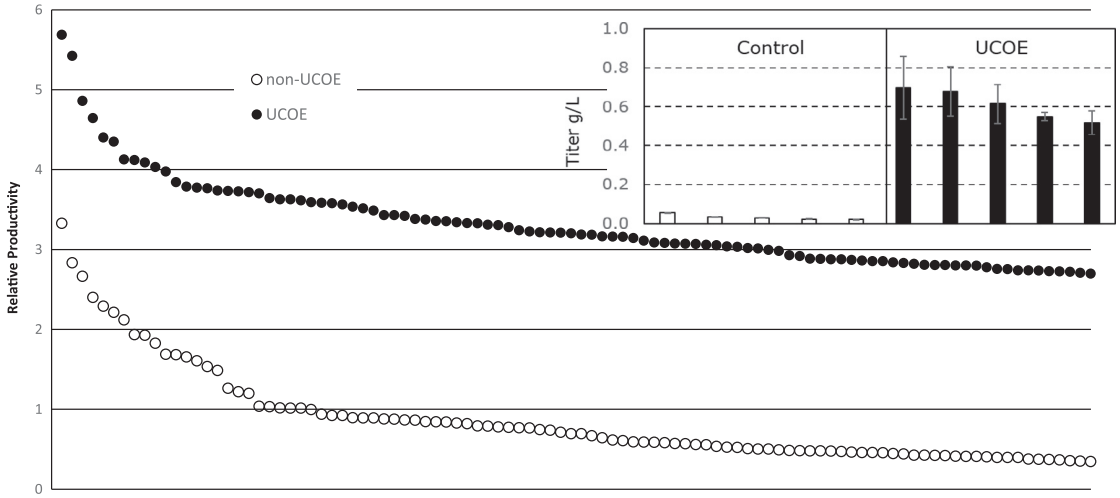

Fig. 3. UCOE enhances the productivity of clonal cells. Bulk pools of stably transfected UCOE vector-derived and control cultures were sub-cloned using limiting dilution. Greater than 500 clones of each were analyzed for antibody titer secreted into the culture medium using an ELISA and analyzed for cell number by flow cytometry. Relative Productivity is represented as the ratio of monoclonal antibody titer divided by the cell number measurement. The inset shows the batch antibody titer of the five highest expressing UCOE-derived and non-UCOE control clones as determined by ELISA.

selection and isolation of highly expressing clones. The usage of UCOEs within biomanufacturing holds promise for expediting upstream biomanufacturing stages by facilitating the production and selection of a candidate clonal cell population in less than three months. However, there is still a requirement to select the most effective UCOE-heterologous (viral) promoter combination for maximum protein therapeutic biomanufacture depending on the nature of the protein to be produced and especially the cell line to be employed.

4. Applications of UCOEs in gene therapy

Gene therapy is the treatment of a disease or medical disorder through the introduction of exogenous therapeutic genetic material into appropriate cellular targets in order to overcome the effects of specific genetic mutations and/or to normalise cellular function. Successful gene therapy therefore relies upon a means to introduce exogenous genetic material in an efficient, target-specific manner, and the stable incorporation and expression of the genetic material from within the target cell for the appropriate period of time.

Lentiviral vectors (LVs) represent one of the most promising gene delivery systems in the field of human gene therapy. LVs offer a reliable means by which exogenous genetic material can be integrated into dividing and non-dividing target cell genomes and their use has shown encouraging outcomes in a number of clinical trials for conditions such as primary immunodeficiencies (Sauer et al., 2014) and leukodystrophies (Aubourg, 2016). Once integrated into the target cell genome the therapeutic transgenes are inherited upon mitosis and in principle can be expressed over long periods. However, natural cellular defence mechanisms mediate the epigenetic silencing of proviral genetic material through DNA methylation, histone deacetylation, and chromatin condensation, acting to diminish or even reverse therapeutic effects (Antoniou et al., 2013). It is therefore important, if gene therapy using LVs is to be effective, that the epigenetic silencing of therapeutic transgenes be prevented by incorporating protective regulatory elements.

The first studies looking at UCOE function within LVs investigated the use of a 2.2 kb A2UCOE sub-fragment (Fig. 4A, fragment A) (Antoniou et al., 2003) linked to reporter genes expressing from the inherent *HNRPA2B1* promoter (Fig. 4B, construct I). In this configuration the A2UCOE was shown to provide higher expression, greater stability, and more reproducible levels of expression per vector copy number than the spleen focus-forming virus (*SFFV*), *hCMV*, and elongation factor-1α (*EF1α*) promoters within *ex vivo* haematopoietic stem cell (HSC) transduction-transplantation experiments in mice (Zhang et al., 2007). The A2UCOE has also been shown to confer sustained expression of an *EGFP* reporter *in vivo* for 10 months, after the transplantation of transduced human fetal liver-derived HSCs into immunocompromised mice, whereas in the same period, expression from vectors driven by *PGK* and *EF1α* promoters showed a 5-fold and 22-fold reduction in expression, respectively (Dighe et al., 2014). Recently the utility of A2UCOE-based LVs has been further extended by the demonstration that they can confer stable, long-term expression following pre-natal delivery to the fetal liver and haematopoietic stem cells (Kao et al., 2016). Furthermore, post-natal haemophilia B curative levels of human FIX were produced following low level delivery of an A2UCOE-FIX LV to the fetal liver (Kao et al., 2016).

The capability of the A2UCOE to resist silencing has been found to be at least in part due to its ability to resist DNA methylation (Zhang et al., 2010). In summary, whereas in the short-term, there is no discernible difference in the levels of expression driven by promoters with and without a linked UCOE due to the time taken for transgene silencing (de Poorter et al., 2007), frequently used promoters from either viruses (*SFFV*, *CMV*) or native genes (*EF1α*, *PGK*) are invariably silenced over a period of weeks or months, resulting in a variegated expression pattern post-transduction and compromised transgene expression (Dighe et al., 2014; Zhang et al., 2010).

The potential utility of UCOEs within a gene therapy context was further extended when it was demonstrated that their linkage to tissue-specific heterologous promoters retains promoter tissue specificity, which frequently demands tissue-restricted therapeutic gene transcription. It has been shown that the 1.5 kb A2UCOE core sub-fragment (Fig. 4A, fragment B) linked to the muscle-specific desmin (*DES*) and granulocyte-specific myeloid-related protein-8 (*MRP8*) promoters (Fig. 4B, construct II) confers stable and appropriate tissue-specific expression (Brendel et al., 2012; Talbot et al., 2010). Furthermore, an A2UCOE-MRP8-gp91phox LV was shown to be able to completely rescue the X-linked chronic granulomatous disease phenotype in a mouse model system following *ex vivo* transduction-transplantation of HSCs at low average vector copy number per cell (Brendel et al., 2012).

The generation of induced pluripotent stem cells (iPSCs) from adult human cells with well-defined reprogramming factors, followed by genetic modification, directed differentiation, and then transplantation into patients, holds great promise as a treatment in regenerative medicine (Karagiannis and Eto, 2016; Takahashi et al., 2007). One potential limitation of this technique is the epigenetic-mediated silencing of therapeutic transgenes introduced into iPSCs, especially upon their differentiation down the desired lineage. Because of this, several investigators have examined the function of UCOEs in this class of stem cells. The 1.5 kb A2UCOE linked to heterologous promoters has been found to consistently confer high and stable gene expression in human and murine iPSCs and in embryonic stem cells before and after differentiation into lineages representative of all three germ layers (Ackermann et al., 2014; Pfaff et al., 2013).

The inherent limited capacity of viral vectors requires that regulatory elements present within the incorporated therapeutic transcription unit must be minimised to allow for maximisation of transgene capacity. Hence, over the past decade various sized sub-fragments of UCOEs, particularly those derived from the A2UCOE, have been built and tested in order to maximise the size available space for linked transgenes (Fig. 4A). Initial studies showed that a 2.2 kb A2UCOE region encompassing both the *CBX3* and *HNRPA2B1* transcriptional start sites and the methylation-free CGI and with transgenes driven directly off the innate *HNRPA2B1* promoter (Antoniou et al., 2003), maintains stable expression in a LV context both *in vitro* and *in vivo* (Zhang et al., 2007). The preferred A2UCOE sub-fragment that confers stability of expression when linked to heterologous ubiquitous and tissue-specific promoters, was the 1.5 kb core element (Fig. 4A, fragment B), which again extends over the alternative first exons of *CBX3* and into the first intron of *HNRPA2B1* (Fig. 4B, construct II) (Brendel et al., 2012; Zhang et al., 2010). A 1.2 kb sub-fragment of this 1.5 kb element, which lacks 300 bp from the *HNRPA2B1* end, was first shown to possess an equal UCOE capability when linked to the highly silencing-prone *SFFV* promoter (Zhang et al., 2010). Uchiyama and colleagues investigated the function of a 0.6-kb A2UCOE sub-fragment that encompasses only the *HNRPA2B1* promoter and 5′ flanking region, but no *CBX3* sequences (Fig. 4A, fragment D). Linked to *EGFP* and the Wiskott-Aldrich syndrome protein gene (*WAS*) (Fig. 4B, construct IV), this element showed stable and long-term expression upon Foamy virus vector transduction into HSCs *in vitro* and *in vivo* comparable to the endogenous *WAS* promoter (Uchiyama et al., 2012). More recently a 0.7 kb A2UCOE-derived sub-fragment incorporating the alternate first exons and promoter of *CBX3* (Fig. 4A, fragment C) was shown to possess *a*nti-silencing functions comparable to that of the 1.5 kb A2UCOE in multipotent and pluripotent stem cells when linked to the *SFFV* and *MRP8* promoters (Müller-Kuller et al., 2015). In addition, expression from the inherent *CBX3* promoter of the 0.7 kb sub-fragment (Fig. 4B, construct III) was also shown to be stable, albeit at very low levels (Müller-Kuller et al., 2015).

Severe combined immunodeficiency (SCID) is a life-threatening group of primary immune deficiencies caused by a number of genetic mutations that result in the abnormal development of lymphocytes. The most common form of SCID, representing 50–60% of cases, is X-linked

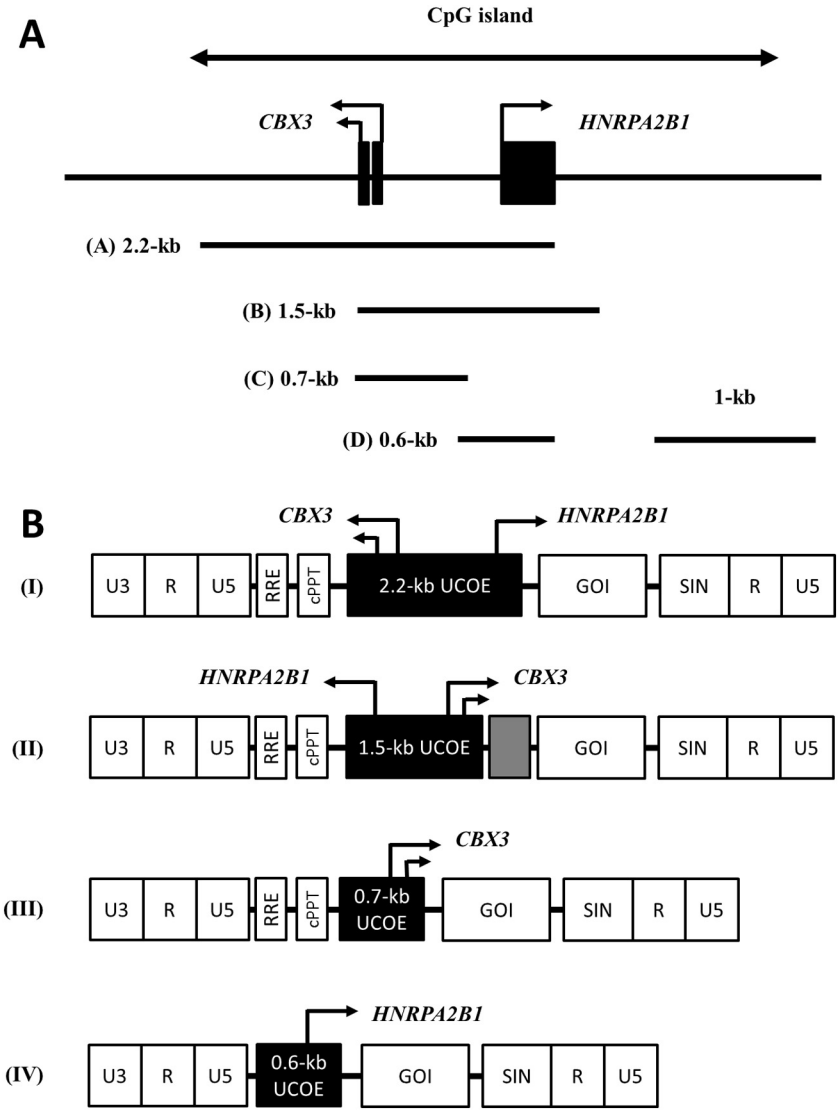

Fig. 4. *HNRPA2B1-CBX3* genomic region and functional A2UCOE sub-fragments. (A) Top panel: illustration of the *HNRPA2B1-CBX3 promoter* and methylation-free CGI region. Rectangles with associated arrows represent the first exons of the two genes and direction of transcription. Lower panel: lines A–D denoting extents and lengths of sub-fragments of the *HNRPA2B1-CBX3* promoter-CGI region showing A2UCOE function. (B) Illustration of lentiviral vectors showing transgenes under control of A2UCOE sub-fragments. Construct I: 2.2 kb A2UCOE driving expression of a gene of interest (GOI) directly from the innate *HNRPA2B1* promoter. Construct II: augmentation of heterologous promoter (grey box) activity by linkage of the 1.5 kb A2UCOE fragment. Note: the 1.5 kb A2UCOE can be replaced with other sub-fragments of this element within this transcription unit design. Construct III: 0.7 kb *CBX3* sub-fragment driving expression from its own promoter. Construct IV: 0.6 kb *HNRPA2B1* promoter fragment driving expression from its innate promoter.

SCID (SCID-X1), which is caused by mutations in the interleukin-2 receptor gene (*IL2RG*) encoding the common γ-chain cytokine receptor subunit (Fischer, 2000). In 2007, Zhang and colleagues achieved efficient full immunogenic reconstitution within a SCID-X1 mouse model system using an LV-A2UCOE-IL2RG cassette based on expression directly off the *HNRPA2B1* promoter (Fig. 4B, construct I) and an *ex vivo* HSC transduction-transplantation procedure at low vector copy number per cell, which was superior to that obtained with an SFFV-IL2RG vector (Zhang et al., 2007).

SCID can also stem from deficiencies of *RAG2*, resulting in the total absence of T and B cells. In a murine *Rag2* knockout model system, incomplete immunogenic reconstitution was achieved with a LV construct containing an *SFFV* promoter driving expression of *RAG2*. Although T cell proliferation and antibody responses, and plasma antibody and T cell receptor levels were restored, subnormal levels of B cells and double-positive T cells were observed. It was concluded that the silencing of the *SFFV* promoter through DNA methylation may have been responsible for the incomplete phenotype reversal. Replacement of the *SFFV* promoter with the 2.2 kb A2UCOE (Fig. 4A, fragment A) resulted in a greater correction of the phenotype, including B cell reconstitution to near normal levels (van Til et al., 2012).

Mutant O^6-methylguanine DNA methyltransferase ($MGMT^{P140K}$) is protective against certain chemotherapeutic agents and has shown clinical potential as a myeloprotective agent in glioblastoma patients treated with temozolomide (Adair et al., 2012). Transduction of myeloid and lymphoid cells with an LV containing an 1.5 kb A2UCOE-*PGK* promoter$MGMT^{P140K}$ construct provided high and stable levels of expression, which allowed for transgenic cell selection and conferred significant myeloprotection against a combination of O^6-benzylguanine and carmustine in a mouse model (Phaltane et al., 2014).

The risks of insertional mutagenesis are reduced through the usage of self-inactivating (SIN) viral vector systems. However, enhancer elements found in internal regulatory regions present within transgenes can still possess mutagenic potential. The A2UCOE is an enhancer-less element and as such vectors incorporating the A2UCOE are at reduced risk of insertional mutagenesis events compared to vectors containing enhancers (Zhang et al., 2007). A second safety consideration is the

elimination of aberrant splicing. A number of studies have reported examples of vector-mediated aberrant splicing, a potential source of vector toxicity (Almarza et al., 2011; Cavazzana-Calvo et al., 2010; Moiani et al., 2012; Montini et al., 2009). Knight and colleagues reported that both native and activated cryptic splice donor sites within the A2UCOE act as sources of aberrant transcripts (Knight et al., 2012). However, targeted point mutations successfully inactivated splice donor sites within the A2UCOE, negating this source of potential insertional mutagenesis (Knight et al., 2012).

In addition to its use within LV systems, the A2UCOE has also been evaluated in a non-viral replicating episomal plasmid vector context. Upon incorporation into a plasmid, the 1.5 kb A2UCOE linked upstream of an *hCMV* promoter stably increased levels of transgene expression (Hagedorn et al., 2013).

The functional characteristics of UCOEs are unprecedented amongst genetic regulatory elements. Compared to other commonly used promoters, the A2UCOE, with expression from either the innate *HNRPA2B1* promoter or when linked to a heterologous promoter, shows greater stability and levels of transgene expression. The A2UCOE also conserves tissue-specific expression and maintains stability of expression in adult, embryonic, and induced pluripotent stem cells before and after differentiation. Thus the A2UCOE is potentially a powerful tool in both LV and episomal plasmid vector gene therapy contexts.

5. Conclusion

The UCOE class of genetic regulatory elements shows broad and well-established utility in protein therapeutic biomanufacturing and encouraging applications in retroviral vector-based gene therapy. In cell lines commonly used for the production of recombinant monoclonal antibodies and other proteins, UCOEs based on the human *HNRPA2B1-CBX3* and murine *Rps3* loci have shown the ability to confer reproducible and high levels of linked gene expression and protein production. In many instances, expression is higher with the UCOE than with standard protocols and expression systems. The utility of UCOEs to markedly expedite the rapid selection of highly-expressing cell clones has also been shown. As such, the UCOE-based gene expression platform shows promise as a means to improve the time and cost efficiency of upstream biomanufacturing stages by increasing the proportion of stable highly-expressing clones and expediting clonal cell line isolation.

The A2UCOE has been the mostly widely investigated UCOE in the field of gene therapy. Alone or linked to heterologous ubiquitous or tissue-specific promoters, the A2UCOE has been shown to confer stable and long-term transgene expression not only in somatic cells, but also in adult, embryonic, and induced pluripotent stem cells, and their differentiated progeny. The A2UCOE has been shown to outperform other commonly used promoter elements over extended time periods, including the *hCMV*, *SFFV*, *EF1α*, and *PGK* promoters. The A2UCOE functions in viral and plasmid vector contexts and functional subfragments of the A2UCOE have been identified, enabling the maximisation of vector transgene capacity. Several studies have shown clinical promise for the A2UCOE as a ubiquitously-acting gene therapy regulatory element where long-term, high expression levels are required with minimal variation and insertional mutagenesis risk.

Conflicts of interest

MNA holds inventor status on patents covering the biotechnological applications of UCOEs and receives income from out-licencing of the UCOE gene expression system and acts in a consultancy capacity to MilliporeSigma. JO and KM are employees of MilliporeSigma, who is the owner of the intellectual property rights to the UCOE gene expression platform. JJN declares that he has no conflicts of interest.

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